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Structure Determination of Macrolactin Compounds with Antibacterial Activities Isolated from *Bacillus polyfermenticus* KJS-2

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In this study, we isolated five macrolactin compounds from a fermentation broth of *Bacillus polyfermenticus* KJS-2. The macrolactin compounds were structurally identified as macrolactin A (MA), 7-*O*-malonyl macrolactin A (MMA), 7-*O*-succinyl macrolactin A (SMA), macrolactin E (ME) and macrolactin F (MF) via a variety of NMR techniques, COZY, DEPT, HMQC and HMBC, and mass and specific rotation assays. The three macrolactin compounds, MA, MMA and SMA, profoundly inhibited the growth of both vancomycin-resistant *Enterococci* (VREs) and methicillin-resistant *Staphylococcus aureus* (MRSA), the inhibition of which were estimated via a disc agar diffusion bioassay. MA, MMA, and SMA exhibited antibacterial activities superior to those of vancomycin and teicoplanin.

Key words : *Bacillus polyfermenticus* KJS-2, macrolactin, antibacterial activity, vancomycin-resistant Enterococcus, methicillin-resistant *Staphylococcus aureus*

Introduction

The macrolactin compounds are a class of macrolide antibiotics, all of which contain a 24-membered lactone ring [8]. They were isolated from several natural sources, an unclassifiable marine bacterium, *Streptomyces*, *Actinomadura,* and *Bacillus* species [4,8,11,16]. Thus far, 26 macrolactin compounds have been structurally elucidated [8,16,21,22,26-28].

Macrolactins evidence a variety of structures, as well as broad pharmacological activities. MA inhibits not only proliferation of murine melanoma cancer cell and *Herpes simplex* virus [8], but also the synthesis of the enzyme squalene synthase [4]. Additionally, MA appears to be capable of protecting neuronal cells against glutamate toxicity [11] and also protects T lymphoblast cells against injury by the human immunodeficiency virus (HIV) [8]. The macrolactin compounds exhibit a marked antimicrobial activity [8-10,14,16, 22,26]. MMA and SMA also inhibit the growth of the dangerous Gram-positive bacteria, MRSAs and VREs [21].

We have been primarily interested in macrolactin compounds and their numerous pharmacological activities, most specifically the antibacterial activities evidenced by some macrolactin compounds against multidrug-resistant bacteria.

Increased resistance to antimicrobial agents and the emergence of multidrug-resistant gram-positive bacterial pathogens, including MRSA and VRE, have become pressing issues in the medical community[17,18,20]. These organisms induce nosocomial infections and are associated with increased rates of morbidity and mortality [5,7,25]. The glycopeptide antibiotics vancomycin and teicoplanin are commonly employed to treat infections caused by MRSA and VRE [19,24]. However, recent emergences of infections evidencing high-level resistance to these glycopeptide antibiotics have resulted in restrictions being placed on their use for the treatment of nosocomial infections [1,3]. Linezolid, a novel synthetic oxazolidinone compound, has been identified as a potential alternative against MRSA and VRE [6,15,29]. However, linezolid-resistant Enterococci have already been reported [2,23]. Thus, the development of new antibiotics is clearly necessary in order to overcome these bacterial infections.

Bispan strains are recognized in the Japanese pharmacopoeia as amylolytic bacilli and commercial probiotic bacteria mixed with at least four strains of *Bacillus polyfermenticus* [13]. In a previous study, we newly isolated *B. poly-*

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fermenticus KJS-2 from these Bispan strains [12].

In the process of surveying the antibacterial activity of secondary metabolites generated by *B. polyfermenticus* KJS-2 against VREs and MRSAs, we identified the five macrolactin compounds as major components evidencing antibacterial activities.

In this paper, we report the isolation, purification, structural elucidation, and antibacterial activities of macrolactin compounds generated by *B. polyfermenticus* KJS-2.

Materials and Methods

Strains and media

The following standard strains were employed in this study: *Enterococcus faecalis* ATCC29212 and *Staphylococcus aureus subsp. aureus* ATCC25923. Clinical isolates of vancomycin-resistant *Enterococcus faecium* (VanA type, VRE-1, VRE-2) and methicillin-resistant *Staphylococci* (MRSA-4, MRSA-9) were obtained from the Dong-A University Medical Center and Kyungsung University, respectively, both of which are located in Busan, Korea. All bacteria were cultivated at 35°C and 200 rpm for 24 hr in tryptic soy broth (TSB, Difco). All organisms were maintained in 25% glycerol solution at -72°C for short-term storage and freeze-dried with 10% skim milk for long-term storage.

Chemicals

Vancomycin, methicillin, and ampicillin were purchased from Sigma. Teicoplanin was obtained from IL-DONG Pharmaceutical Co., LTD. Antibiotic stock solutions were freshly prepared in sterile distilled water. Stock solutions of MA, MMA, SMA, ME and MF were freshly prepared in methanol.

Culture and extraction

B. polyfermenticus KJS-2 was used to produce macrolactins. The strain was grown in TSB medium, which functioned as the seeding medium. The seeding medium (280 ml) was cultured at 37°C on a rotary shaker at 200 rpm until the culture reached an OD_{600} of 0.7, and then inoculated into a fermentor (10 l, Biotron, Korea) containing 7 l of fermentation medium. The fermentation medium consisted of 16 g of nutrient broth (Difco), 2.5 µM $FeSO_4$, 500 µM $CaCl_2$, 10 µM $MnCl_2$, 1 mM $MgSO_4$, 13 mM KCl, and 10 g of skim milk in a total volume of 1 L.

To produce MA, MMA, ME and MF, fermentation was conducted at 30°C with an agitation rate of 200 rpm and an aeration rate of 1.0 l/min. The pH was maintained at 6.8 via the addition of 2N H_2SO_4 and 3N NaOH.

The fermentation broth was extracted three times with an equal volume of ethyl acetate. The ethyl acetate layer was evaporated under vacuum. The residue was dissolved in methanol.

To generate SMA, the seeding culture (40 ml) was inoculated into 1 l of TSB medium supplemented with 50 ml of HP-20 resin. Following 2.5 days of incubation at 30°C and 200 rpm, the culture broth was filtered to collect HP-20 resins. The collected resins were then washed for 1 hr with 250 ml of ethyl acetate, and this procedure was repeated three times. The eluted solvent was evaporated under vacuum, and the final residue was dissolved in methanol.

Medium pressure liquid chromatography (MPLC) separation

For the partial purification of a macrolactin compound, the extract was fractionated with a Buchi MPLC system (Buchi pump C-605, column 1.5×23 cm, fraction collector Buchi C-660) using LiChroprep C-18 (40~63 µm, Merck) as an adsorbent. The MPLC conditions were as follows: 40% acetonitrile or 40% acetonitrile containing 20 mM ammonium acetate was maintained at a flow rate of 15 ml/min for the first 800 sec, after which 100% methanol was maintained with a flow rate of 20 ml/min from 800 to 1200 sec on an MPLC chromatograph. Elution was monitored at 262 nm.

Each of the fractions was evaporated *in vacuo* and extracted with ethyl acetate and water. The ethyl acetate layer was evaporated under reduced pressure, after which the residue was dissolved in methanol.

Semi-preparative HPLC separation

To achieve higher purity, each of the MPLC fractions was injected into a semi-preparative liquid chromatography apparatus (Young-Lin Co. Ltd., Korea) equipped with a Gemini C18 column (250 by 10 mm, Phenomenex Co., Ltd.). 40% acetonitrile solution was maintained at a flow rate of 5ml/min. The pooled fractions were evaporated under vacuum and extracted with ethyl acetate and water. The ethyl acetate layer was evaporated under reduced pressure, and a pale yellow or off-white powder was subsequently generated. Each of the powders was stored at -20°C.

Conditions for HPLC and LC/Mass analysis

HPLC and LC/Mass analysis were conducted using a Shimadzu class vp and Agilent 1100 seriesapparatus, respectively. The Agilent 1100 series contains a high pressure liquid chromatograph connected to an online diode array detector (DAD) and a mass selective detector (MSD) equipped with an electrospray ionization chamber. A Zorbax SB C18 column (dimensions, 250 by 4.6 mm) was used for HPLC and LC/Mass analysis. The mobile phase consisted of acetonitrile and water including 0.1% formic acid. The acetonitrile concentration increased in a linear fashion from 0 to 100% for 20 min. The UV wavelength was set at 262 nm. The flow rates of the mobile phase in HPLC and LC/Mass were 1.5 and 1 ml/min, respectively. For LC/Mass analysis, the flow was directly introduced into the ESI interface. The capillary voltage was set to 4 kV, the drying gas temperature was 300°C, the dry gas flow was 12 l/min, and the nebulizer pressure was set to 50 psi.

Structure determination

For nuclear magnetic resonance (NMR) spectroscopy, 30 mg of the purified compound was dissolved in DMSO-d_6. ^{1}H-NMR, selective homonuclear-decoupled, ^{13}C-NMR, DEPT-45, DEPT-90, DEPT-135, ^{1}H-^{1}H COZY, HMQC and HMBC spectra were recorded using a Brucker Advance DRX 500 spectrometer operating at 500 MHz. For specific rotation measurements, 40 mg of the compound was dissolved in 1 ml of MeOH. The solution was transferred into the tube (length=100 mm, volume=1 ml), after which the specific rotation was measured using a POLAX-D polarimeter (Atago, Germany).

Antibacterial activities by agar diffusion bioassay

A cell suspension was overlaid on a cation-adjusted Mueller-Hinton agar plate (5×10^5 cfu/plate). Sterile discs (6 mm AA discs, Whatman) impregnated with 10 μl of the antibiotic stock solution or the sample stock solutions (the final compound concentration on the disc, 50 μg) were placed on the agar plates. 10 μl of MeOH was employed as a control. All bacteria were incubated for 18 hr at 35°C. After incubation, the diameters (mm) of the inhibition zones were measured with a ruler.

Results

MPLC fractionation and antibacterial activities

The fermentation broth was extracted with ethyl acetate, and subsequently equally divided into three fractions by MPLC. Each of the MPLC fractions was then analyzed via LC/Mass, and their anti-VRE and anti-MRSA activities were assessed via a paper disc-agar diffusion bioassay. The large inhibition zones of bacterial growth were observed around the discs. Interestingly, both of the second and third MPLC fractions strongly inhibited the growth of VREs and MRSAs. Table 1 provides their anti-VRE and anti-MRSA activities. The UV spectra and mass data resulting from the LC/Mass analysis of two MPLC fractions suggested that their major components might be macrolactin compounds. Fig. 2 shows LC chromatograms of the crude extract and the three fractions divided by MPLC.

Production, isolation, and structure determination of five macrolactin compounds

Five major compounds were purified from a fermentation broth of Bp2. The production yield of compound I was estimated as 58 mg/l. The purity was 98.3% on the HPLC chromatogram observed at a UV wavelength of 262 nm. Compound I evidenced a 425.4 $[M+Na]^+$ and 441.4 $[M+K]^+$

Table 1. Antibacterial activities of the crude extract and three fractions divided by MPLC

Strain	Class of bacteria	Inhibition diameter (mm)			
		Crude extract	MPLC fractions		
			1st	2nd	3rd
E. faecalis ATCC29212	Gram positive	12	-	12	8
VRE-1	Gram positive	15	-	14	13
VRE-2	Gram positive	17	-	17	14
S. aureus ATCC25923	Gram positive	25	13	25	23
MRSA-4	Gram positive	30	16	30	30
MRSA-9	Gram positive	33	23	33	32

E. faecalis ATCC29212, *Enterococcus faecalis* ATCC29212; VRE, vancomycin-resistant *Enterococcus faecium* *S. aureus* ATCC25923, *Staphylococcus aureus* ATCC25923; MRSA, methicillin-resistant *Staphylococcus aureus*
See materials and methods for experiment procedures.

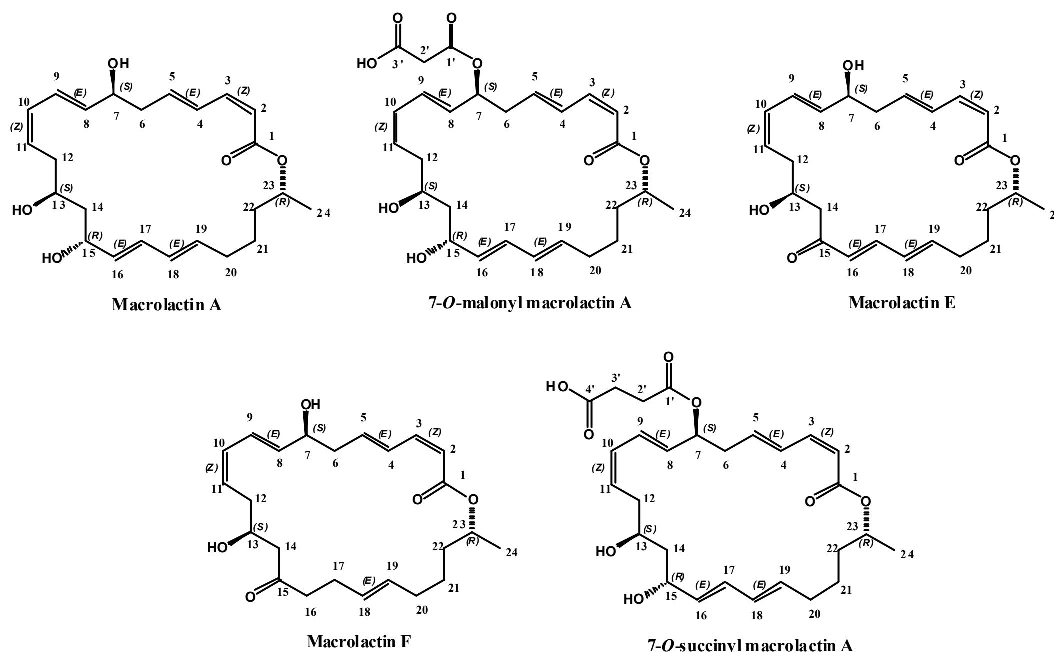

Fig. 1. Chemical structures of five macrolactin compounds produced from *B. polyfermenticus* KJS-2.

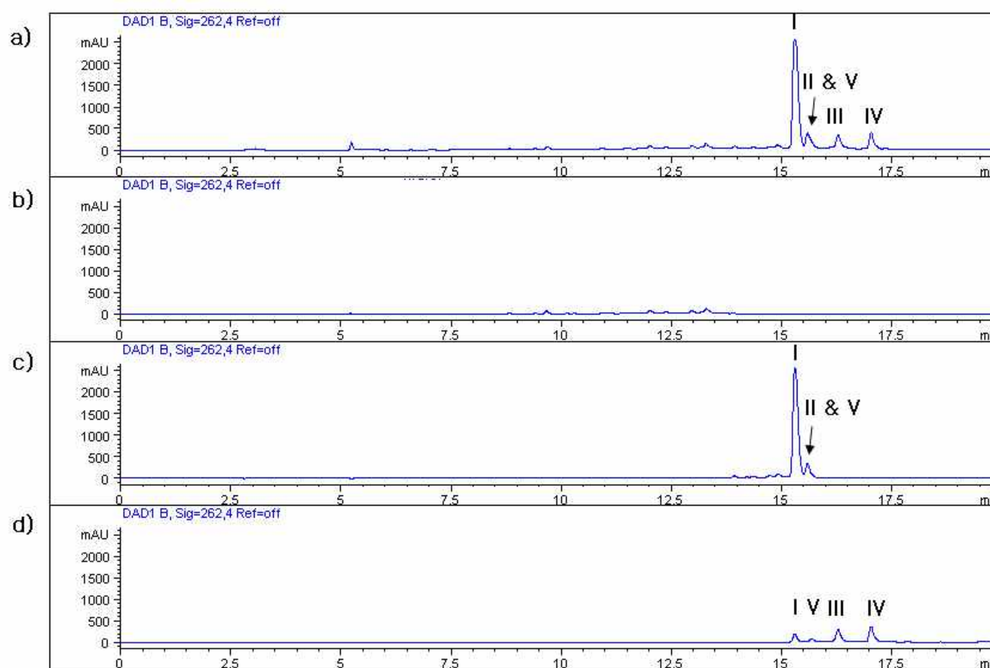

Fig. 2. LC chromatograms of the crude extract and three fractions divided by MPLC. a), LC chromatogramsof the crude extract, b), c) and d) 1st, 2nd and 3rd fraction, respectively. Roman numerals indicate the peaks which were identified as macrolactins. LC chromatogram was generated at 262 nm of UV wavelength.

mass-to-charge ratio in the (+) ESI-mass spectrum. The maximal absorptions observed were at 230 and 262 nm in the UV spectrum. Compound I was identified as a monocyclic compound composed of $C_{24}H_{34}O_5$, via analysis of the ^1H-^1H COZY, HMQC, and HMBC NMR spectra (Table 2). However, these data were not sufficient to elucidate the structure because the ^1H NMR spectrum included complex peaks. For this reason, a selective homonuclear decoupling

Table 2. ^{1}H NMR data (500 MHz) of compounds 1-5 in DMSO-*d6*[a]

Carbon No	1	2	3	4	5
2	5.55 (1H, d, 11.68)	5.58 (1H, d, 11.38)	5.55 (1H, d, 11.50)	5.54 (1H, d, 11.53)	5.56 (1H, d, 11.47)
3	6.65 (1H, dd, 11.54, 11.51)	6.62 (1H, dd, 11.34, 11.92)	6.66 (1H, dd, 11.50, 11.51)	6.65 (1H, dd, 11.51, 11.55)	6.63 (1H, dd, 11.48, 11.51)
4	7.06 (1H, dd, 11.56, 15.13)	7.1 (1H, dd, 11.74, 15.65)	7.07 (1H, dd, 11.49, 15.12)	7.11 (1H, dd, 11.36, 15.26)	7.09 (1H, dd, 11.63, 15.1)
5	6.19 (1H, dt, 15.17, 14.68)	6.1 (1H, dt, 15.23, 14.62)	6.21 (1H, m)	6.2 (1H, dt, 15.35, 14.93)	6.08 (1H, dt, 15.15, 14.71)
6	2.32 (2H, m)	2.54 (2H, m)	2.32 (1H, m) 2.25 (1H, m)	2.33 (2H, m)	2.56 (2H, m)
7	4.16 (1H, m)	5.39 (1H, m)	4.08 (1H, m)	4.12 (1H, m)	5.38 (1H, m)
8	5.71 (1H, dd, 5.23, 15.16)	5.72 (1H, dd, 5.68, 15.41)	5.71 (1H, dd, 6.07, 15.18)	5.71 (1H, dd, 6.12, 15.26)	5.71 (1H, dd, 5.48, 15.24)
9	6.48 (1H, dd, 15.06, 11.21)	6.53 (1H, dd, 15.26, 11.08)	6.34 (1H, dd, 15.12, 11.2)	6.34 (1H, dd, 15.24, 11.1)	6.47 (1H, dd, 15.15, 11.17)
10	6.02 (1H, dd, 10.90, 10.85)	6.04 (1H, dd, 11.33, 10.63)	6.06 (1H, dd, 11.2, 10.96)	6.04 (1H, dd, 11.05, 11.05)	6.05 (1H, dd, 11.08, 10.5)
11	5.49 (1H, m)	5.59 (1H, m)	5.47 (1H, m)	5.45 (1H, m)	5.59 (1H, m)
12	2.36 (1H, m) 2.14 (1H, m)	2.4 (1H, m) 2.17 (1H, m)	2.28 (2H, m)	2.26 (2H, m)	2.41 (1H, m) 2.17 (1H, m)
13	3.64 (1H, m)	3.67 (1H, m)	4.02 (1H, m)	3.96 (1H, m)	3.64 (1H, m)
14	1.42 (2H, m)	1.4 (2H, m)	2.71 (1H, dd, 6.48, 15.25) 2.53 (1H, m)	2.44 (2H, d, 6.51)	1.42 (2H, m)
15	4.14 (1H, m)	4.16 (1H, m)			4.15 (1H, m)
16	5.49 (1H, dd, 6.15, 14.85)	5.52 (1H, dd, 6.04, 14.62)	5.99 (1H, d, 15.56)	2.38 (2H, t, 7.745)	5.52 (1H, dd, 6.12, 14.52)
17	6.04 (1H, dd, 14.84, 10.35)	6.03 (1H, m)	7.08 (1H, m)	2.1 (2H, m)	6.06 (1H, dd, 14.12, 10.63)
18	5.96 (1H, dd, 10.50, 14.62)	6.0 (1H, dd, 10.58, 14.52)	6.24 (1H, m)	5.35 (1H, m)	6.0 (1H, dd, 10.53, 14.99)
19	5.59 (1H, dt, 14.36, 14.18)	5.57 (1H, m)	6.23 (1H, m)	5.36 (1H, m)	5.59 (1H, dt, 14.52, 13.85)
20	2.07 (2H, m)	2.07 (2H, m)	2.26 (1H, m) 2.14 (1H, m)	1.94 (2H, m)	2.07 (2H, m)
21	1.44 (2H, m)	1.54 (1H, m) 1.41 (1H, m)	1.44 (2H, m)	1.36 (2H, m)	1.45 (2H, m)
22	1.52 (2H, m)	1.56 (1H, m) 1.42 (1H, m)	1.61 (2H, m)	1.53 (2H, m)	1.54 (2H, m)
23	4.9 (1H, m)	4.93 (1H, m)	4.95 (1H, m)	4.92 (1H, m)	4.94 (1H, m)
24	1.2 (3H, d, 6.27)	1.2 (3H, d, 6.27)	1.22 (3H, d, 6.25)	1.19 (3H, d, 6.27)	1.2 (3H, d, 6.27)
1′					
2′		3.39 (2H, s)			2.47 (2H, -)
3′					2.50 (2H, -)
4′					

[a]The assignments were aided by 1H-1H COZY, DEPT, HMQC, HMBC, and selective homonuclear decoupled spectra.

technique, which is a very effective method of determining the coupling constants more accurately, was utilized for analysis of the ^{1}H NMR spectrum, thereby determining the geometric configurations (data not shown). Furthermore, the ^{13}C NMR spectrum (Table 3) evidenced an ester carbonyl resonance at 165.89 ppm and 12 methine carbons between 67.14 and 143.82 ppm assigned to six double bonds (Table 3). As a result, compound I was identified as a macrolactin compound. The specific rotation was shown to be -10 (C=4.0 in MeOH) at 17°C this is similar to the reported rotation of MA [8,11,21]. Based on these data, compound I was identified as MA (Fig. 1).

The production yield of compound II was 16 mg per liter of fermentation broth. The purity was 84.88% on the HPLC chromatogram observed at a UV wavelength of 262 nm. Compound II displayed not only mass-to-charge ratios of 511.7 $[M+Na]^+$ and 487.7 $[M-H]^-$ in the ESI-mass spectra, but also maximal absorptions of 230 and 258 nm in the UV spectrum. The NMR spectra of compound II were nearly identical to those described by Romero-Tabarez *et al.,* in that the 7-H signal was shifted approximately 1.2 ppm downfield [21]. The ^{13}C signals equivalent to the malonyl residue were identified at 166.47 and 168.27 ppm, and the direction of specific rotation was identified as levorotatory (-). These data, together with the ^{1}H-^{1}H COZY, HMQC and HMBC NMR spectra, helped to define the structure of compound II as MMA (Fig. 1, Table 2-3).

Compounds III and IV evidenced peaks at 424.0 $[M+Na]^+$

Table 3. ^{13}C NMR data (125 MHz) of compounds 1-5 in DMSO-*d6*[a]

Carbon No.	1	2	3	4	5
1	165.89 C	165.75 C	165.61 C	165.84 C	165.28 C
2	117.04 CH	117.88 CH	116.61 CH	117.05 CH	117.35 CH
3	143.82 CH	143.3 CH	143.38 CH	143.86 CH	142.82 CH
4	128.49 CH	129.35 CH	128.0 CH	128.63 CH	128.88 CH
5	142.73 CH	139.72 CH	142.01 CH	141.88 CH	139.50 CH
6	42.23 CH2	38.74 CH2	41.29 CH2	41.68 CH2	38.37 CH2
7	70.02 CH	73.64 CH	70.24 CH	70.55 CH	72.23 CH
8	137.86 CH	131.03 CH	137. 56 CH	137.91 CH	130.96 CH
9	124.01 CH	126.93 CH	124.03 CH	124.48 CH	126.09 CH
10	129.91 CH	129.26 CH	130.07 CH	130.32 CH	128.78 CH
11	128.16 CH	130.26 CH	127.22 CH	127.65 CH	129.63 CH
12	35.86 CH2	35.8 CH2	34.75 CH2	35.28 CH2	35.27 CH2
13	67.14 CH	67.07 CH	67.0 CH	67.0 CH	66.67 CH
14	43.82 CH2	43.81 CH2	45.83 CH2	49.32 CH2	43.54 CH2
15	67.59 CH	67.53 CH	198.94 C	209.17 C	67.13 CH
16	136.41 CH	136.48 CH	128.80 CH	43.06 CH2	135.98 CH
17	128.63 CH	128.62 CH	143.28 CH	26.69 CH2	128.19 CH
18	130.68 CH	130.67 CH	129.08 CH	129.42 CH	130.19 CH
19	133.46 CH	133.31 CH	144.85 CH	130.52 CH	132.88 CH
20	31.82 CH2	31.74 CH2	31.82 CH2	31.84 CH2	31.33 CH2
21	24.49 CH2	24.49 CH2	23.82 CH2	24.92 CH2	24.08 CH2
22	34.74 CH2	34.72 CH2	34.50 CH2	34.99 CH2	34.29 CH2
23	70.57 CH	70.61 CH	69.96 CH	70.33 CH	70.17 CH
24	19.97 CH3	19.96 CH3	19.55 CH3	20.14 CH3	19.50 CH3
1′		166.47 C			171.30 C
2′		42.02 CH2			28.61 CH2
3′		168.27 C			28.78 CH2
4′					173.18 C

[a]The assignments were aided by 1H-1H COZY, DEPT, HMQC, and HMBC spectra.

and 426.0 $[M+Na]^+$ m/z, respectively, in the (+) ESI spectrum. Additionally, compound III evidenced maximum absorbance at 264 nm, whereas compound IV evidenced maximum resonance at 262 nm. The purities of III and IV were 98.9% and 90.8%, respectively, on the HPLC chromatogram observed at a UV wavelength of 262 nm. The production yields of compounds III and IV were 3 and 5 mg per liter of fermentation broth, respectively. The NMR data of compound III was nearly identical to that of MA, except for the absence of a 15-H resonance, the appearance of 16-H as a doublet at 5.99 ppm (d, J=15.56 Hz) and a downfield shift of the protons (16-H, 17-H, 18-H and 19-H) in the ^{1}H-NMR spectrum (Table 2). Our comparison of the ^{13}C NMR spectra of compound III and MA suggested that they differed only in terms of the presence of ketone in compound III (Table 3). The structure of compound III was identical to that of ME, based on the totality of the data [8]. The NMR data of compound IV were nearly identical to those of ME, except for the appearance of 14-H as a doublet at 2.44 ppm (d, J=6.51 Hz), the existence of 16-H as a triplet at 2.38 ppm (t, J=7.745 Hz) in the ^{1}H NMR spectrum, a downfield shift of the 15-C (209.17 ppm) and the appearance of two additional aliphatic methylenes in the ^{13}C NMR spectrum. The NMR spectra of compound IV together with the LC/Mass data helped to define the structure of compound IV as MF (Fig. 1) [8].

The production yield of compound V was 138 mg per liter of fermentation broth. Compound V evidenced a purity of 97.02% on the HPLC chromatogram, 525.6 m/z of [M+Na]+ in the (+) ESI spectrum, 501.6 m/z of [M-H]- in the (-) ESI spectrum, and maximum absorbance at a UV wavelength of 258 nm. The specific rotation was -15 (C=4.0 in MeOH) at 17°C. The NMR data of compound V were nearly identical to those of MMA except for the appearance of 2′-H (2.47 ppm), 3′-H (2.5 ppm), 1′-C (C, 171.3042), 2′-C (CH2, 28.6109), 3′-C (CH2, 28.7757) and 4′-C (C, 173.1817) signals, which reflected the existence of succinyl residues. The NMR spectra and the specific rotation value of com-

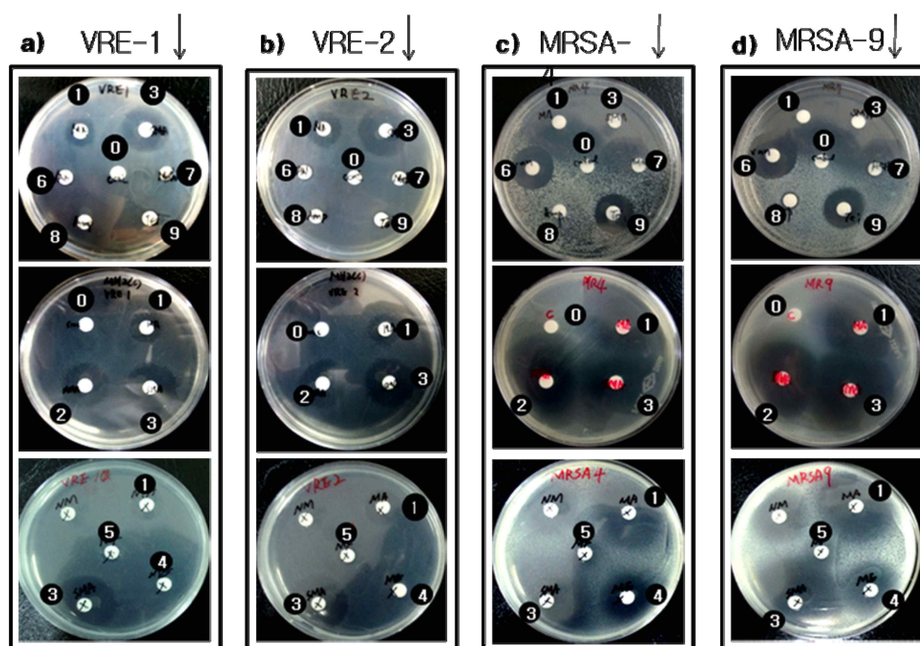

Fig. 3. The growth inhibition zones of five macrolactins and reference compounds against VREs and MRSAs. a) and b) indicates the growth inhibition of VRE-1 and VRE-2 (5×10^5cfu/plate) treated with the test compounds, respectively. Each of c) and d) indicates the growth inhibition of MRSA-4 and MRSA-9 (0.5~5×10^5cfu/plate) with them. 0, MeOH as a control; 1, macrolactin A; 2, 7-*O*-malonyl macrolactin A; 3, 7-*O*-succinyl macrolactin A; 4, macrolactin E; 5, macrolactin F; 6, vancomycin; 7, methicillin; 8, ampicillin; 9, teicoplanin. Each disc is compromising 50 µg of the test compounds. VRE1/2, vancomycin-resistant *Enterococcus faecium*-1/2. MRSA-4/9, methicillin-resistant *Staphylococcus aureus*-4/9.

pound V were almost identical to those of SMA [10]. The LC/Mass data and the NMR data showed that compound V was an SMA [10].

Antibacterial activities of the five macrolactin compounds

The antibacterial activities of the purified macolactin compounds against reference strains and clinical isolates were evaluated using a paper disc agar diffusion bioassay (Table 4) and representative results are shown in Fig. 3. All five of the macrolactin compounds inhibited the growth of *S. aureus*, MRSA-4 and MRSA-9 and three of them (MA, MMA, and SMA) inhibited the growth of *E. faecalis*, VRE-1, and VRE-2. Furthermore, the antibacterialactivities of MA, MMA,and SMA against both VRE and MRSA were superior to those of vancomycin and teicoplanin.

Discussion

We purified five macrolactin compounds from a fermentation broth of BP2 [12] and elucidated their structures. The macrolactin compounds were MA, MMA, SMA, ME and MF. The antibacterial activities of the macrolactin compounds against reference strains and clinical isolates were evaluated via a paper disc agar diffusion bioassay. Three macrolactin compounds, MA, MMA,and SMA (but not ME and MF) inhibited the growth of both MRSA and VRE. ME and MF proved active against MRSA, but were not active against VRE. The principal structural difference between MA/MMA/SMA and ME/MF was the presence of the C-15 hydroxyl group. Nagao *et al.* reported that the hydroxyl group at C-15 might perform an important function in the antibacterial activity of macrolactin compounds [16]. Our results also suggest that the C-15 hydroxyl group of macrolactins could exert an effect on the antibacterial activity. We attempted to determine the MIC value for the macrolactins, but they did not completely inhibit the growth of the strains via the agar diffusion method. The same results were reported by Romero-Tabarez *et al.* [21]

The production levels of MA, MMA, SMA, ME, and MF were 58 mg, 16 mg, 138 mg, 3 mg and 5 mg per L of fermentation broth, respectively. The production yield of macrolactins has generally been approximately 3 mg/l, according to the reports published thus far [8,10,11,14,16,21,27]. Based

on these results, *B. polyfermenticus* KJS-2 is apparently a potentially valuable and effective strain for the production of macrolactin compounds.

The three macrolactin compounds--MA, MMA, and SMA--evidenced profound antibacterial activity against the clinical isolates of MRSA and VRE, and their levels of activity were superior to those of vancomycin and teicoplanin. The potential activity of macrolactins should be evaluated by *in vivo* experiments in the future, owing to the somewhat ambiguous MIC data this will be our next topic of inquiry.

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초록 : *Bacillus polyfermenticus* KJS-2에서 분리된 항생물질 마크로락틴류의 구조결정

김동희[1]·강경란[1]·김현우[2]·윤시열[3]·김천규[3]·토쿠타로 야마구치[4]·송재경[4]·강재선[5]*
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바실러스 폴리퍼멘티쿠스 케이제이에스-2의 발효액에서 5 종류의 마크로락틴을 분리하였다. 각각의 구조를 분석한 결과로 마크로락틴 에이, 말로닐 마크로락틴 에이, 숙시닐 마크로락틴 에이, 마크로락틴 이, 마크로락틴 에프 등으로 규명되었다. 특히 3종의 항생제인 마크로락틴 에이, 말로닐 마크로락틴 에이, 숙시닐 마크로락틴 에이는 반코마이신 내성 장구균과 메치실린 내성 황생포도상구균에 효과적으로 성장억제를 나타내었다. 이것은 반코마이신이나 테이코플라닌보다 우수한 항생효과를 보였다.